AD-A178 755 HUMAN HYBRIDOMAS FOR EXOTIC ANTIGENS(U) SALK INST FOR BIOLOGICAL STUDIES LA JOLLA CA DEVELOPMENTAL BIOLOGY LAB M COHN 01 OCT 86 DAMD17-85-C-5266 1/1

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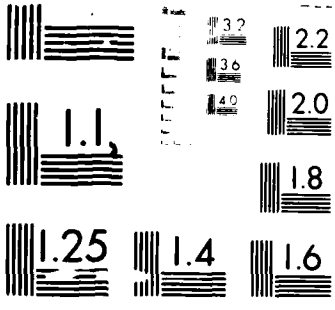

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Human Hybridomas for Exotic Antigens

Annual Report

Dr. Melvin Cohn, Ph. D.

Developmental Biology Laboratory

October 1, 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5266

The Salk Institute for Biological Studies

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AD-A178755

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188 Exp. Date: Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: The Salk Institute for Biological Studies; 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code): 10010 N. Torrey Pines Road, La Jolla, CA 92037	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5266
8c ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO 61102A; PROJECT NO 3M1-61102BS12; TASK NO AC; WORK UNIT ACCESSION NO 125

11 TITLE (Include Security Classification)

(U) Human Hybridomas for Exotic Antigens

12 PERSONAL AUTHOR(S)

Cohn, Melvin

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 9/1/85 TO 8/31/86	1986 October 1	17

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

Human hybridomas: Monoclonal antibodies: Exotic antigens

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

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20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a NAME OF RESPONSIBLE INDIVIDUAL: Virginia M. Miller	22b TELEPHONE (Include Area Code): 301/663-7325; 22c OFFICE SYMBOL: SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

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SUMMARY

This is the first annual report under a 3-year contract to develop methods for generating human hybridomas *in vitro* that can produce antibodies specific for exotic antigens which could not be produced by normal *in vivo* immunization procedures. The basic methodology for generating human hybridoma cell lines was available at the outset of the contract. However, several minor modifications were required in order to adapt methods for use with peripheral blood lymphocytes (PBL) obtained by lymphapheresis of normal human donors. Much of this first years work has been directed at establishing optimal conditions for recovering hybridomas from PBL. The methods now in hand allow the recovery of 5 stable hybridomas per 10^6 PBL; given that 10% of PBL are B-cells and roughly 10% of B-cells can be captured as hybridomas, this means that for every $2x10^3$ potential B-cell targets that are recoverable in principle, one is obtained. Considering the 10^3-fold excess of irrelevant cells present during the fusion process, a recovery of 1 hybrid for every $2x10^3$ potential targets makes the next step of the project fully feasible.

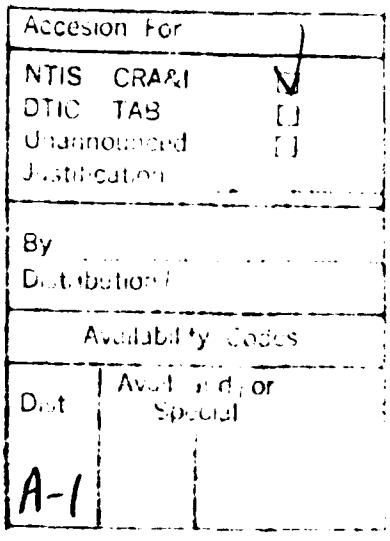
Although keenly sought after, primary *in vitro* immunization methods for use with PBL remain to be developed. While a considerable effort has been made to generate primary antibody responses *in vitro*, we aim to stop short of this mature stage of B-cell differentiation because antibody-producing cells fail to form good, stable hybridomas. Our aim is to activate resting B-cells and initiate cell division without allowing making the B-cells undergo terminal differentiation; early post-induction B-cells are nearly ideal for making hybridomas. Although we remain confident that sufficient activated B-cells can be produced via *in vitro* immunization to manufacture hybridomas specific for exotic antigens, we have not overlooked the advantages that would follow from producing a hybridoma library from immune vaccinated or convalescent individuals exposed to important, otherwise lethal, viral infections. Since the additional effort required to establish hybridoma libraries from immune individuals is minimal, we have explored some aspects of this methodology using "normal" immunity to Diphtheria Toxin and patients suffering from autoimmune disease. In all of these *in vivo* immune donor examples we have tried as far as possible to utilize the collaborative interests of others to carryout the time consuming screening of hybridoma supernatants.

FOREWORD

Citations of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research Council (DHEW Publication No. (NIH)78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45FR46.



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1. Objectives.

The overall goal of the work being carried out under this contract is the development of methods for generating human hybridomas specific for highly toxic exotic antigens. Of primary interest is the development of methods that will enable human B-lymphocytes to be immunized *in vitro* in a way suitable for fusion with the B-cell tumor WI-L2-729-HF_2, thereafter enabling the capture of stable antibody-producing hybridomas. Included in the broad aims of this contract is the production of human hybridomas from immune donors, particularly in the case of volunteers immunized with attenuated vaccines and known to be producing protective antibodies against viruses of major military significance.

2. Background.

There is no doubt that primary *in vitro* immunization of human B-cells and their capture as hybridomas represents a major technical advance and has considerable military significance. With this technology in hand it would be possible to generate antibodies against highly toxic pathogens that would otherwise be lethal *in vivo*. An exception would be that rare class of agents which lethally infect B-lymphocytes.

A survey of the literature over the past 10 years reveals no substantiated examples of successful primary *in vitro* immunization with human B-cells. There are numerous reports showing that following *in vivo* priming, an *in vitro* boost can be given and antibody-producing cells recovered; this, however, is of no special interest in the context of our central aim. The available evidence from our own previous studies with human and murine B-cells all points to the strong likelihood that mature plasma B-cells have terminally differentiated and cannot form immortal hybrids. At some stage prior to terminal differentiation, and after induction, B-cells can be captured as antibody-producing hybridomas. Thus, from a practical point of view, it is necessary to assay for *in vitro* antigen activation of B- cells by hybridoma formation, not by antibody secretion. This means that rare, specifically activated, B-cells which might occur at a frequency of around 10^{-6} must be selectively enriched by at least 10^2, preferably 10^3-fold if the task of screening thousands of hybridomas is to be avoided. Moreover, as our experience with PBL derived hybridomas has now shown, no more than $2x10^5$ total cells in 0.1ml culture wells can be plated without impairing the recovery of hybrids. This means that it is insufficient to merely increase the probability that a hybridoma will be of interest because irrelevant cells which seem to "clog" the HAT selection must also be eliminated.

For comparative purposes it might be helpful to comment on our results with WI-L2-729-HF_2 (refs. 1-2) and those of the Sloan Kettering group (refs. 3-5) which are based on the NS-1 murine tumor parent. Contrary to our experience with murine B-cell tumors, the Sloan Kettering group has reportedly been able to obtain stable human immunoglobulin production with hybridomas generated with NS-1. Similar to our experience, they find the LICR-2 human lymphoblastoid line to be much less efficient than NS-1. While we obtain fusion efficiencies in the range of 1 to $5x10^{-6}$ with WI-L2-729-HF_2, the Sloan Kettering group using NS-1 has obtained a fusion efficiency of about $6x10^{-6}$. However, while the WI- L2-729-HF_2 hybridomas are greater than 80% immunoglobulin secretors, and more than 90% remain stable secretors, producing 5μg/ml antibody, the NS-1 hybridomas initially comprise 52% secretors and of these 60% can be

expected to remain stable secretors producing 0.5μg/ml antibody. To a first approximation, the WI-L2-729-HF_2 and NS-1 hybridomas are essentially equivalent, with an edge in favor of WI-L2- 729-HF_2 because of better stability, or in favor of NS-1 because it produces hybrids that are EBNA negative. Since expression of the EBNA antigen is of more theoretical than proven practical concern, we have not worked hard to make the NS-1 murine system as good as is claimed by the Sloan Kettering group.

3. Rationale.

Given the obvious risks in developing a method for *in vitro* immunization, we have arranged our program of work to include the use of B-cells from immune donors for the generation of hybridomas. Usually B-cells from lymphoid organs are used when attempting to recover immune B-cells as hybridomas. While this is feasible in the case of experimental mice (using spleen or lymph nodes) and humans with disease (using tonsil), it is inappropriate to consider the use of surgical intervention in order to obtain immune B-cells from "normal" healthy individuals; it is, however, acceptable to remove aliquots of peripheral blood to obtain lymphocytes (PBL). Therefore, we have made a major, almost exclusive, effort to obtain the best results with PBL B- cells, either recovered from blood, or by lymphapheresis.

Three prototype PBL donors have been selected, 1) "normal" individuals immune to Diphtheria Toxoid (but not recently boosted), 2) autoimmune individuals under treatment for Myasthenia Gravis by plasmapheresis and/or lymphapheresis, and 3) normal individuals "tolerant" to their own hormones (e.g. CRF, corticotrophin-releasing factor). Individuals immune to Diphtheria toxoid represent randomly boosted (by natural infection) examples which are easily screened by ELISA assay and can be used further to study *in vitro* boosting methods for manipulating *in vitro* responses of B-cells. The use of individuals with Myasthenia Gravis was dictated by the availability of "free" screening for antibody via collaborations with other members of our laboratory; these represent chronically immune individuals who are making relatively high levels of antibody (in the order of 10μg/ml in serum). These two classes of immune individual provide the benchmark standards against which we can assess the likelihood of recovering rare B-cells following *in vitro* immunization. Then, there is the most severe challenge to our technology, the ability to generate a strictly *de novo* immune response to a self component.

As soon as we are confident of our methods for recovering as hybridomas the B-cells of immune individuals, it will be appropriate to draw upon the vaccinated individuals available to the Army and convert their protective antibodies to immortal hybridomas. The effort required to carry-out this aspect of our goals represents a very small addition to the basic studies that would have to be carried out before being able to fully and accurately assess *in vitro* immunization methods

While considerable attention has been given to the hybridoma techniques we have not overlooked developments in the use of serum-free media, the use of B-cell growth and differentiation factors, and the preparation of "universal" helper and killer cell lines. Some preliminary work has been carried out in all of these areas as summarized in the results section, but without a fully optimized fusion system to test these various reagents, it has been considered premature to devote a lot of time and effort to these areas.

4. Experimental Methods.

4.A Fusion method.

4.A.i Preparation of PEG:

Weigh 1g of PEG (MW 4000 from EM Science) into a sterile glass tube and melt the PEG in a boiling water bath, then transfer to 56° water bath. Prepare fresh a 25% (v/v) solution of DMSO in RPMI 1640 at room temperature. Add 1ml of 25% DMSO to 1g of molten PEG to make 50% (w/v) PEG and 12.5% (v/v) DMSO. Transfer the 50% PEG to a 37° water bath ready for use.

4.A.ii Preparation of cells:-

WI-L2-729-HF_2 tumor cells are harvested at $\cong 10^6$ cells per ml and washed twice in RPMI 1640. Lymphocytes after appropriate pre-culture treatments, are harvested, washed twice in RPMI 1640 and then counted.

4.A.iii Fusion:-

Using a 50ml conical centrifuge tube, mix up to $2.5x10^7$ lymphocytes and $2.5x10^7$ WI-L2-729-HF_2 cells and pellet; the procedure can be scaled down to using as few as 10^6 of each cell type. To the pelleted cell mixture 2ml of PEG/DMSO is added and gently swirled to get the cells into suspension. After 7min at room temperature, over about 30sec add 50ml of RPMI 1640 and gently pellet the cells. Resuspend the pellet in 20ml of R20F medium (or to about $2x10^6$ total cells per ml) and incubate in 100mm plastic tissue culture dishes overnight at 37° in an atmosphere of 5% CO_2 in air.

4.A.iv Preparation of R20F medium:-

To 90ml of RPMI 1640 add:

1ml of 1M HEPES buffer.
1ml of 100mM sodium pyruvate.
1ml of 100x non-essential amino acids.
1ml of Fungi-Bact (Irvine Scientific)
150µl of 50mg/ml gentamicin.
2.5ml of 80mM glutamine.
20ml of FCS from pre-screened batches.
10µl of 2-mercaptoethanol (0.5M stock).

4.A.v Plating of hybridomas for selection in HAT:-

After overnight incubation, cells are plated at $2x10^5$ cells per 0.1ml in 96 well trays. Next day (48hr post fusion) each well receives 0.1ml of 2X HAT. At weekly intervals until clones are macroscopically visible, HAT is changed weekly. In wells with vigorously growing clones of hybridomas the medium is changed to HT around 4 weeks post fusion and one week later the medium is further changed to RPMI 1640 supplemented with 10% FCS.

HAT is prepared from a 100x HT supplement (supplied by Whittaker M.A. Bioproducts) and 100x Aminopterin (Sigma) which is prepared by dissolving 1.76mg of aminopterin in 100 ml of water.

4.A.vi Stimulation of lymphocytes in culture:-

4.A.vi.a Fetal calf, Human A/B and serum-free media

Fetal calf serum is pre-screened to support a high cloning efficiency of WI-L2 72/ HF_2 and hybridomas; lot-to-lot variation is considerable and usually about 1 in 10 is suitable. Several different suppliers have been tested with no overall superiority of any one.

Human AB serum pools are obtained from Irvine Scientific and Whittaker M.A. Bioproducts.

Serum-free media; three versions have been tested with two commercially available (HB101, Hanna Biologicals and Nutricyte, J. Brooks Laboratories), and one "home-grown" version developed in our laboratory by Dr. M. Gersten (ref. 5).

4.A.vi.b Pansorbin (PSN) and reformalinized Pansorbin (rfPSN).

As supplied by Calbiochem, Pansorbin is a 10% (w/v) suspension of heat inactivated and formalin fixed *S. aureus* (Cowan I strain) cells in PBS. For stimulation of PBL, Pansorbin is diluted to a final concentration of 0.015% as determined by titrating each batch (no significant variation has been noted in three different batches).

To reduce the shedding of cell wall components, the reformalinization of Pansorbin has been recommended. This is accomplished by resuspending the pellet of a 5ml aliquot of Pansorbin in 5ml of 1.5% formaldehyde and incubating at room temperature for 1hr. Cells are then washed twice in 50ml of PBS and reconstituted to 5ml (i.e., a 10% suspension).

4.A.vi.c Lymphocyte culture conditions.

Lymphocytes (PBL or tonsil) are cultured in RPMI 1640 supplemented with 10% (v/v) serum or in serum-free media at a concentration of $2x10^6$ cells per ml in 100mm plastic tissue culture dishes at 37° with an atmosphere of 5% CO_2 in air. When appropriate, Pansorbin or reformalinized Pansorbin is added to a final concentration of 0.015%. In experiments designed to evaluate the effect of mononuclear phagocytes (which are present in large numbers in PBL) adherent cells are removed by incubating cells on plastic tissue culture dishes for 2hr at 37° and removing the non- adherent cells (mostly lymphocytes) by gently swirling the plates and decanting the non-adherent cells.

As supplied, lymphapheresis cells (Hemacare) have a significant number of erythrocytes remaining. To remove erythrocytes from PBL (whole blood or lymphapheresis packs) cells are centrifuged over Ficoll-Paque (supplied by Pharmacia); briefly, 35ml of whole blood or lymphapheresis packs diluted with two volumes of saline are layered on 15ml of Ficoll-Paque and centrifuged for 40 min at 2200 rpm, PBL are removed from the interface and washed twice in 50ml of RPMI 1640.

4.A.vii Screening of hybrids for immunoglobulin isotypes:-

Plates of the 96 well type (Falcon #3911) are sensitized after removing static with a damp paper towel. A 1:200 dilution of goat anti-human kappa (supplied by TAGO) or a 1:700 dilution of goat anti-human lambda (supplied by TAGO) in PBS-AE (phosphate buffered saline containing 0.0065% azide and 0.04% EDTA) is used to sensitize plates. The sensitization is carried out overnight at 4°. Plates are then washed thoroughly with

deionized water, followed by one wash with PBS-AE. The plates are then blocked by adding 0.1ml of 1% (w/v) BSA (bovine serum albumin, supplied by Sigma) in PBS-AE and allowing the plates to stand for 45min at room temperature. Plates are then washed twice with deionized water.

To appropriately sensitized plates 45μl of antibody (usually hybridoma culture supernatants) is added and plates allowed to stand overnight at 4°. Next morning plates are warmed to room temperature, the supernatants drained, and plates washed thrice with deionized water then twice with PBS-AE and allowed to drain.

After plates have bound antibodies present in the culture supernatants, 50μl of appropriately diluted goat anti-human immunoglobulin that has been coupled with alkaline phosphatase is added and the plates allowed to incubate for 3-4 hours at 37°. Plates are then washed thrice with deionized and distilled water then twice with PBS-AE and allowed to drain.

To determine the amount of human immunoglobulin bound to the plates 50μl of nitrophenyl phosphate substrate is added and the plates allowed to stand overnight at 4°; if the amount of human antibody bound to plates is very high, and the wells rapidly turn yellow, plates are read earlier. The amount of nitrophenol released is measured photometrically using an automated plate reader (Dynatech) illuminated with 405nm wavelength light.

The goat anti-human immunoglobulin is conjugated with alkaline phosphatase as purchased (TAGO) and diluted 1:20,000 in 1% BSA in PBS-AE. The nitrophenyl phosphate substrate is prepared as a 1mg/ml solution in 0.05M sodium bicarbonate buffer pH9.8.

4.A.viii Screening of hybridomas for Diphtheria Toxoid (DT) binding:-

Essentially the same technique as described for assaying immunoglobulins is used to assay the binding of hybridoma culture supernatants to Diphtheria Toxoid sensitized plates; the amount DT used to sensitize plates was determined using normal human sera as a positive control, typically 1-10μg/ml was adequate. Sensitization procedures have since been further refined using both positive and negative hybridoma culture supernatants. The Diphtheria Toxoid was obtained from the Massachusetts Public Health Laboratory.

4.A.ix Establishment of murine T helper and killer cell lines:-

Balb/c mice were immunized by intraperitoneal injection of Ficoll purified human PBL ($2x10^7$ cells per 0.5ml). Mice were boosted at monthly intervals, each time with cells from a different donor. After the second boost the spleen from one mouse was removed and cultured in DMEM (10% FCS) at a concentration of 10^6 mouse cells/ml and $2x10^6$ mitomycin C treated human cells per ml. The human donor last used for *in vivo* boosting was used again for the first *in vitro* boost. After 10-14 days cells were harvested, counted and boosted.

4.A.x Production of B-cell growth factors:-

Essentially three different sources of factors have been tested. The reference source of a mixture of factors was obtained from a Pokeweed mitogen stimulated tonsil lymphocyte population. Cells were cultured at a density of $2x10^6$ cells per ml in RPMI 1640 supplemented with 10% FCS or Human AB serum (no significant differences were

noted with these two serum sources) and 10μg/ml mitogen (this amount of mitogen is determined by titration of each batch). Culture supernatants are harvested after 48hr by centrifuging out the cells and filtering the supernatant prior to storage at -20°. PWM supernatants are routinely used at 10% concentration in assays.

Supernatants from two cloned cell lines have been prepared in bulk and used in preliminary tests. The MOW10 cell line was obtained from Dr. L. Mayer (presently at the Mount Sinai Medical School) and the RPMI 1788 B-cell line was obtained from the American Type Culture Collection. Cells were seeded at $2x10^6$ per ml in RPMI 1640 supplemented with 10% FCS and grown to saturation (about 4 days). Supernatants were harvested by centrifuging out the cells and filtering the supernatant which was then stored frozen at -20°.

4.A.xi Selection of nonsecreting WI-L2-729-HF_2 variants:-

Work has been proceeding to select a non-secreting variant of WI-L2-729-HF_2 by multiple rounds of mutagenesis and selection by assaying clones for the absence of surface immunoglobulin.

5. Results.

5.A Evaluation of basic cell preparation and fusion parameters.

The results summarized below were sought because the initial PBL B-cell fusions using lymphapheresis to obtain lymphocytes were very erratic. Over a roughly 6 month period we were able to identify several important variables and then establish standardized procedures which now ensure that variability is minimal.

5.A.i Source of PBL:-

The lymphapheresis technique is applied by a specialized California State licensed company (Hemacare) which uses pre-screened and paid donors to obtain various blood cells and products. By carefully monitoring the ratio of lymphocytes to mononuclear phagocytes and determining the fusion efficiency of cells we were able to establish criteria that the lymphapheresis operator could use to select the optimal cell fraction for our studies. It should be noted that the lymphapheresis operators have no objective criteria to select a given fraction and they must visually assess the color of the selected fraction; this is presently characterized as the "rose-pink" layer in their jargon.

5.A.ii Fusion conditions:-

Previously, conditions for optimal recovery of hybridomas had been determined using tonsil lymphocytes and these conditions were initially used in the present work with PBL B-cells. Although some of our initially erratic results could be attributed to the lymphapheresis methods, the fusion method itself seemed to be a significant source of variability. Several parameters were examined including the source and preparation of PEG, the temperature and duration of exposure of cells to PEG, the concentration of DMSO, and the stage of growth of the WI-L2-729-HF_2 tumor parent. Accordingly, the modified fusion protocol is as follows:

5.A.ii.a Preparation of PEG fusogen.

PEG MW 1500 (supplied by J.T. Baker) or PEG MW 4000 (supplied by EM Science) were found to be reproducibly adequate when several batches of each were tested for toxicity. Providing the pH of PEG 1500 was adjusted to 7.5 - 8.0 with HEPES some hybridomas could be recovered; however, higher fusion efficiencies and better reproducibility was obtained with PEG 4000 and this is now used routinely.

PEG is sterilized by placing it in a boiling water bath for 30min, then cooling to 56°. To obtain a final concentration of 50% PEG and 12.5% DMSO in the fusion reaction, the initial PEG solution is made at 50% by adding an equal volume of freshly prepared 25% DMSO in RPMI 1640. At the time of DMSO addition the PEG solution is moved to a 37° degree water bath where it is kept until ready for use.

5.A.ii.b Preparation of tumor cells.

The WI-L2-729-HF_2 cells must be harvested in mid log growth at a concentration of between $5x10^5$ and $2x10^6$ cells per ml. It is important to observe this limitation. Cells taken too early in growth or if allowed to exceed $2x10^6$ cells per ml can be a major source of erratic fusion results.

5.A.ii.c Cell fusion conditions.

Exposure of cell mixtures to PEG and DMSO is best carried out at room temperature for 6-8 min rather than 3-4 min at 37°. At the higher temperatures time seems to be a very critical factor affecting the total recovery of hybrids; by working at room temperature timing is less critical.

Ideally the lymphocyte to tumor cell ratio should be close to 1:1; at ratios greater than 2:1 the fusion efficiency drops significantly and at less than 1:1 there is no significant improvement. As an aside we note that when attempting to capture small numbers of activated B-cells in the presence of an excess of resting B-cells the effective lymphocyte to tumor ratio will be much less than 1:1.

5.A.ii.d Recovery of hybridomas.

The fetal calf serum used to grow hybrids in selective media is pre-screened for a high cloning efficiency of WI- L2-729-HF_2 and established hybrids; when the cloning efficiency at one cell per well is 60%, the particular batch of FCS can be reasonably assured of providing adequate support for the growth of emergent hybrids.

5.A.iii Stimulation of lymphocytes:-

5.A.iii.a Effects of different media for cell culture.

As shown in the summary fusion data (Table I), there is a low fusion efficiency when PBL are fused without pre- culture; on average the fusion efficiency is 10-fold or more below the frequency obtained when PBL are first cultured for 3 to 5 days in various media. Although there is some variation with different donors, FCS and Human AB serum supplements are essentially equivalent in raising the fusion efficiency to about $3x10^{-6}$ from less than $3x10^{-7}$ when PBL are fused directly. Our previous experience with tonsil B- cells clearly pointed to an essential role of Pansorbin to increase the fusion efficiency. However, experience with PBL now shows quite clearly that Pansorbin is not essential. It is unlikely that the serum is acting as a significant source of antigen during the 3-5 day culture period because both FCS and Human AB sera are similarly stimulatory and the serum-free Nutricyte medium is clearly not less stimulatory (in fact Nutricyte is strongly stimulatory). Moreover, in excess of 500 hybridomas have been

TABLE I

Summary of Fusions

Donor	Cells	cultured in	Days	clones /. 10^6cells
7	PBL	FCS	4	3.0
7	PBL	FCS/rfPSN	4	12.0*
7	PBL	FCS/PSN	4	1.4
7	PBL	FCS	5	5.2
7	PBL	FCS/rfPSN	5	4.2
7	PBL	FCS/PSN	5	5.6
7	PBL	FCS/PSN	6	3.0
7	PBL	FCS	4	0.3**
7	PBL	FCS	5	0.3**
7	PBL	FCS/rfPSN	5	0.1**
7	PBL	FCS/PWM	5	0.4**
MG-1	PBL	NONE	0	0.3
MG-1	PBL	FCS	4	2.6
MG-1	PBL	FCS/rfPSN	4	0.3
MG-1	PBL	FCS/PSN	4	2.3
MG-1	PBL	NONE	0	0.01
MG-2	PBL	NONE	0	0.3
8	PBL	FCS	5	2.5
8	PBL	FCS/PSN	5	0.2
8	PBL	FCS	3	3.0
8	PBL	FCS/rfPSN	3	0.1
8	PBL	HuAB	3	0.5
8	PBL	HuAB/rfPSN	3	3.6
8	PBL	NONE	0	0.02
9	PBL	NONE	0	0.1
9	BL	HuAB	5	3.4
9	PBL	HuAB/rfPSN	5	2.0
9	PBL	SFM	5	15.0
9	PBL	SFM/rfPSN	5	3.0
A	Tonsil	FCS	4	10.0
A	Tonsil	FCS/rfPSN	4	4.3
A	Tonsil	FCS/PSN	4	25.0
A	Tonsil	FCS	5	13.0
A	Tonsil	FCS/rfPSN	5	26.0
A	Tonsil	FCS/PSN	5	31.0
A	Tonsil	FCS/PSN	6	30.0

* = Un-explained high fusion efficiency.

** = Used PEG 1500 at pH 7.5.

screened for antibody to FCS and none have been found positive. The question now is how to reduce the background of B-cell blasts so that new blasts obtained during primary *in vitro* immunization will not be lost among a vast excess of irrelevant hybridomas. Thus, it is anticipated that considerable efforts will have to be brought to bear on removing the background activated B-cells prior to *in vitro* immunization and fusion.

A special comment is warranted on the use of serum-free media (SFM) because we had previously found that some media formulations markedly reduced the background fusion efficiency. First, however, it should be noted that while HB101 provides good growth support for hybridomas, it offers little advantage in growing PBL during periods of time required for *in vitro* immunization; likewise the "home- grown" serum-free formula we further adapted from the original murine formulation to attain the correct osmolarity for human cells. Our first experiments with Nutricyte looked particularly encouraging as PBL cultured in this medium for 5 days gave fusion efficiencies of less than 10^{-8} while Nutricyte supplemented with FCS gave the expected 3- $5x10^{-6}$ fusion efficiency. However, subsequently it was found that we had received a Nutricyte medium that had been formulated for mouse cells and when we re-tested the correct human formulation, the fusion efficiency jumped to $1.5x10^{-5}$. We are now in contact with the supplier of Nutricyte and attempting to determine the difference between the two formulations in the hope that a key factor can be pinpointed to explain the dramatic differences we observe; our best guess at present is the high levels of insulin present in Nutricyte.

5.A.iii.b Effect of mitogen (Pansorbin).

We have found consistently that reformalinized Pansorbin is in some way interfering with the stimulation of B-cells from PBL; in contrast, reformalinized Pansorbin is an excellent mitogen when used with tonsilar B-cells. When used directly, Pansorbin has either a mild stimulatory effect or no effect on the fusion efficiency. In some experiments we did note that the Pansorbin did increase the frequency of Ig^+ hybrids, but this effect was only seen when the parallel cultures without Pansorbin (i.e., cultured with serum alone) were atypically low with with respect to Ig^+ hybrids.

5.B Screening of hybridomas for specific antigen binding.

A total (to date) of 434 hybridoma supernatants from five independent fusions and three different donors (PBL donors 8, MG-1 and MG-2) have been screened for binding to Diphtheria Toxoid coated plates. Two hybridomas have been identified from independent fusions in repeated testing that bind to Diphtheria Toxoid. Although the numbers are relatively small at present, a frequency of 0.5% positive hybridomas among Ig^+ clones is well within the range expected for murine systems which do not use a secondary *in vitro* boosting step.

Hybridomas from a single fusion were obtained with cells from a Myasthenia Gravis patient who was undergoing plasmapheresis prior to thymectomy and who had a high titre of serum antibody; this fusion yielded a total of nearly 400 Ig^+ clones. These were screened for binding to purified acetylcholine receptor and one positive clone was identified. There is some doubt that the assay used in this screening was sufficiently sensitive because the acetylcholine receptor used for the assay is isolated from fetal calf, and this source of receptor shows only 1/10th the activity with immune human sera when compared with human receptor preparations. Moreover, there is some question whether the

B-cells producing antibody to the acetylcholine receptor are present in significant numbers in blood, given that large numbers of plasma cells are present in the thymus of patients at this late stage of disease. One potentially important result from this study has been that we can use the PBL obtained from one liter of blood to generate in the order of 1000 hybridomas; this should be sufficient to capture several useful hybridomas from individuals vaccinated with a typical viral vaccine. It is unlikely that we will pursue work with these Myasthenia Gravis individuals since it appears that specific hybridomas are rare in autoimmune disease.

5.C Some preliminary data on the evaluation of B-cell growth factors.

Despite a large number of publications describing a variety of B-cell growth and differentiation factors, there is remarkably little consistency from one laboratory to another. We have written requesting many of the "cloned cell lines", but most were unavailable (excuses abounded!). One set of T-cell hybrids were, however, obtained from Dr. L. Mayer (Rockefeller University) and they performed essentially as claimed. The results obtained in one pilot experiment are summarized in TABLE II below.

Table II

PFC per $5x10^6$ input lymphocytes

antigen	medium	+ PWM factors	+ MOW10 factors
-SRBC	140	220	180
+SRBC	135	680	410
(effect)	(-5)	(+460)	(+230)

Using tonsil B-cells and sheep erythrocytes (SRBC) as antigen in an assay system that had been set up for other studies, we tested the effect of a Pokeweed mitogen (PWM) supernatant and the factors secreted by the most potent of the Mayer lines (MOW10).

Two ml culture wells were seeded with $5x10^6$ tonsil lymphocytes and 10^8 SRBC along with a 1/10 dilution of the two factors. After 5 days direct IgM plaques were enumerated.

Another source of growth factors which may be important has been described in arious reports and is produced by many EBV transformed B-cells. In particular we obtained the 1788 cell line which constitutively produces a factor that enhances B-cell blasting in the presence of sub-optimal concentrations of Pansorbin (1/10th normal). We assayed in a qualitative way the restoration of B-cell blast numbers when PBL were stimulated with 0.001% Pansorbin. The addition of 1788 culture supernatants (25%) for the full 6 days completely restored blasts from around 1% to near 50%. The addition of supernatant for the first three or the last three days of culture had little effect.

Although many more sources of growth factors remain to be tested, and none have yet been tested in the B-cell fusion assay, it seems likely that during the next 12 months much of the present confusion in this new area of research will be sorted out.

5.D Murine T-cell lines specific for common HLA antigens.

The objective in these experiments was to generate effector T-cells from mice that were specific for human HLA Class I or Class II antigens common to all or most haplotypes. In principle we would develop "universal" helper and killer/suppressor cell lines that could be used independent of the antigen specificity of the human B-cells and

without regard to restriction specificity (i.e., MHC haplotype). By repeatedly immunizing mice *in vivo* and *in vitro* with different human donor lymphocytes we expected to select for murine T-cells specific for the HLA Class I or Class II antigens common to all (most) MHC haplotypes.

Initially six Balb/c mice were injected intraperitoneally with 10^7 PBL in 0.5ml of saline; this we refer to as Donor #1. Four weeks later all mice received a further 10^7 PBL from Donor #2. One mouse was sacrificed six days later and spleen cells obtained. These murine spleen cells were put into culture at 10^6 cells per ml and $2x10^6$ PBL from Donor #2 were added after treatment with Mitomycin C. Cells were cultured in HEPES buffered DME supplemented with 10% FCS. After culture for 14 days cells were harvested and re-seeded with fresh PBL from Donor #2 and DME supplemented with 10% FCS. Three weeks later, of the $5x10^6$ murine cells seeded, $3.75x10^6$ were recovered. However, when these cells were boosted with PBL from Donor #3 there was little evidence of vigorous cell growth and one month later fewer than 10^5 cells were recovered after a second boost with PBL from Donor #3. Thus, these murine cells were discarded.

A second mouse was sacrificed 7 days after receiving a third boost with PBL from Donor #3. After being boosted 5 times at 14 day intervals with PBL from Donors #3, #4, and #5. This cell line showed good growth in the presence of mitomycin treated cells from Donors #3 and #4, but this could not be sustained with cells form Donor #5. When attempts to "rescue" the line were made with cells from Donor #5 supplemented with IL-2 (cells from Donors 3 and 4 were no longer available), there was little effect and the line was discarded.

The third attempt to establish a murine T-cell line was begun after the fourth *in vivo* boost with cells from Donor #4. This cell line grew poorly at every passage and was discarded.

This line of work was put aside as experiments with fusions from immune PBL donors began working well and generated an enormous number of hybridomas to maintain and assay.

5.E Selection of non-secreting WI-L2-729-HF_2 variants.

The WI-L2-729-HF_2 cell line expresses surface IgM and traces of IgG of the kappa light chain isotype. To what extent these immunoglobulin chains contribute to the total secreted antibody of hybridomas is not known, though we suspect it is quite significant. In order to eliminate any effects from these immunoglobulin chains we have continued working towards the isolation of a non-producing variant of WI-L2-729-HF_2..

Following multiple rounds of selection on anti- immunoglobulin-coated plates, a variant subclone of WI-L2-729-HF_2 has been isolated which expresses undetectable amounts of heavy chain and a trace amount of kappa. However, there is some question as to the stability of this H-chain loss variant as evidenced by the gradual reappearance of H-chain secretion during 3 months of continuous culture. Efforts are underway to stabilize this clone and select another more stable variant by use of various frameshift mutagens.

6. Discussion and Conclusions.

Progress has been slower than anticipated due to a variety of small technical difficulties encountered as the basic hybridoma technology developed for use with tonsil B-cells has been translated into the PBL B-cell system. While these difficulties have been time-consuming and essential to solve, at no stage has any doubt been cast on the principles underlying this project.

We are now in a position to begin negotiations for the supply of immune donor lymphocytes from individuals vaccinated against some militarily important pathogens. The hybridoma system as it stands can be expected to yield practical results in terms of providing therapeutically useful hybridomas from immune individuals.

The major aim of obtaining a method for *in vitro* immunization and capture of induced B-cells as hybridomas remains ahead of us. High on our priority is to test several methods likely to provide *in vitro* boosting of B- cells specific for Diphtheria Toxoid. As soon as the remaining hybridomas have been screened for anti-DT binding (we have close to 2000 available) we will have a solid baseline figure for the frequency of anti-DT hybridomas prior to *in vitro* manipulation and, therefore, we will be able to readily interpret differences in the frequency of recoverable anti-DT clones.

During the coming year we will begin work on testing various methods for reducing the background (spontaneous) activated B-cells in PBL that make up a substantial proportion of hybridomas following culture. Highest on the priority list are the use of BUdR and light to kill dividing cells during an initial 3 day culture period. Next is the reinitiation of experiments to produce cloned murine helper and killer cell lines specific for common HLA antigens; the killer lines are of key importance as a means to reduce the nonspecific activated B-cells; the helper lines will be crucial to provide a source of inductive factors which, in the presence of antigen, will activate resting B-cells that would not otherwise form hybridomas.

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